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Evidence that the Packaging Signal of Moloney Murine Leukemia Virus Extends into the *gag* Region

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Replication-competent retroviruses can be modified to carry nonviral genes. Such gene transfer vectors help define regions of the retroviral genome that are required in *cis* for retroviral replication. Moloney murine leukemia virus has been used extensively in vector construction, and all of the internal protein-encoding regions can be removed and replaced with other genes while still allowing production of virions containing and transmitting the altered retroviral genome. However, inclusion of a portion of the *gag* region from Moloney murine leukemia virus markedly increases the titer of virus derived from these vectors. We determined that this effect was due to more efficient packaging of the vector RNA into particles and did not depend on protein synthesis from the *gag* region. We conclude that the retrovirus packaging signal extends into the *gag* region. We have found that retroviral vectors containing the complete packaging signal allow more efficient gene transfer into a variety of cell types. In addition, these results may help explain why many oncogenic retroviruses have retained *gag* sequences and often express transforming proteins that are *gag-onc* hybrids.

Retroviral vectors have many advantages as gene transfer vehicles (3, 17), including high efficiency of gene transfer and colinear insertion of transferred genes into host cell chromosomes. Construction of retroviral vectors involves placement of gene(s) to be transferred between viral long terminal repeats (LTRs) and short regions adjacent to the LTRs which are involved in viral replication. For vectors based on Moloney murine leukemia virus (MoMuLV), essentially all of the viral protein-encoding regions can be deleted with retention of a transmissible vector. A region adjacent to the 5′ LTR has been shown to be important for packaging of viral RNA into virions and must be included in retroviral vectors (13, 14). Development of retrovirus packaging cell lines allows production of these replication-defective viruses in the absence of helper virus, thus preventing spread of the vector after the initial infection (14, 16, 26). These systems have proven useful for gene transfer into cultured cells and into animals.

We have observed that certain vectors allow production of virus at a much higher titer than do previous designs. In addition, these new vectors have allowed efficient infection of several potential target tissues for gene therapy, including bone marrow (7, 9, 10, 12) and skin fibroblasts (20). For example, we have demonstrated gene transfer into human hemopoietic progenitor cells with these new vectors (9), whereas we were unable to conclusively demonstrate transfer using previous vector designs (R. A. Hock and A. D. Miller, unpublished results). A similar result was obtained for infection of canine hemopoietic progenitor cells (12). Gene transfer into human skin fibroblasts has been detected with previous vector designs, but the new vectors dramatically increase the efficiency of transfer (20).

Examples of the new more efficient vectors include N2 (1), which contains the neomycin resistance gene, SDHT (19), which carries a mutant dihydrofolate reductase gene, and LHL (20), which carries the hygromycin B resistance gene. The common feature of the new vectors which distinguishes them from earlier vectors is the inclusion of *gag* sequences from the parental helper viruses; therefore, we will call them gag^+ vectors. Reasons for the effectiveness of gag^+ vectors might involve better expression of transferred genes after infection or more efficient participation of the vector in the viral life cycle. For example, a *trans* activator of the Rous sarcoma virus (RSV) promoter has been localized to the *gag* region of RSV (2), and if such a protein was made by gag^+ MoMuLV vectors, this could result in more efficient gene expression after virus infection, leading to a higher apparent titer. Alternatively, the boundaries of the signal required for packaging of viral RNA into virions have not been mapped precisely and could extend into the *gag* region. The presence of the complete signal in the *gag*-containing vectors might allow for more efficient packaging of viral RNA into virions.

We analyzed the effect of inclusion of a portion of the *gag* region in retroviral vectors containing selectable markers. The primary effect of inclusion of *gag* sequences was to increase the amount of vector RNA secreted by virus-producing cells. This effect was not due to increased production of vector RNA in the virus-producing cells and by several criteria was unrelated to potential gene products expressed from the *gag* region. These results suggest that the packaging signal for MoMuLV extends into the *gag* region.

MATERIALS AND METHODS

Cell culture. Cells were grown in Dulbecco modified Eagle medium with high glucose (4.5 g/liter) supplemented with 10% calf serum (ψ-2 cells) or 10% fetal bovine serum (all other cell lines). Previously described cell lines included NIH 3T3 TK^- (31), PA317 (ATCC CRL 9078) (16), PA12 (18), and ψ-2 (14). PA317 retrovirus packaging cells were either from an early passage of the cell line or were reselected in HAT medium (30 μM hypoxanthine, 1 μM amethopterin, 20 μM thymidine) before use. These procedures were used because we found that the percentage of high-titer clones that could be isolated after transfer of a retroviral vector into PA317 cells decreased as a function of the age of the cells. Selection of old PA317 cells in HAT medium fully restored their packaging ability, presumably by

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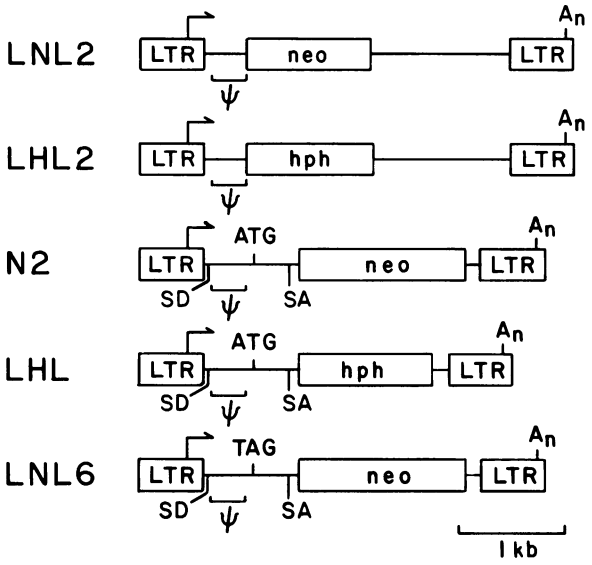

FIG. 1. Retrovirus vectors. The arrows indicate promoters. A_n, Polyadenylation site; SD, splice donor; SA, splice acceptor; kb, kilobase.

killing cells that had lost the thymidine kinase gene and cotransfected viral DNA used to make the cell line (16). PA317 cells were maintained in culture and used for up to 1 month before the cells were reselected or a new aliquot was thawed.

Retrovirus vector construction. The vectors are depicted in Fig. 1. The MoMuLV sequence numbers are as previously described (27). The N2 vector was made (10) by inserting the *neo* gene (4) into a proviral clone of MoMuLV in place of MoMuLV sequences from bases 1039 to 7673 (1). LHL (20) is identical to N2 except that the *neo* gene is replaced with the *hph* gene (8). LNL2 (19) contains the *neo* gene inserted into an MoMuLV-based vector in place of MoMuLV sequences from bases 569 to 6536. LHL2 is identical to LNL2 except that it contains the *hph* gene. The packaging signal in LNL2 and LHL2 is actually derived from Moloney murine sarcoma virus (MoMSV), but this signal is closely related to that of MoMuLV (27). In addition, the normal MoMuLV splice donor (MoMuLV base 206) in LNL2 and LHL2 was removed by alteration of the consensus AGGT sequence to AGGC. The plasmid pLNL contains a virus that is essentially identical to the N2 virus but contains fewer restriction sites in the DNA surrounding the virus than does pN2 to simplify insertion of other genes into the virus. The virus LNL-XHC is identical to LNL except that a 90-base-pair sequence containing a *Hin*dIII site and having *Xho*I and *Cla*I termini was inserted between the *Xho*I and *Cla*I sites of pLNL which lie between the *neo* gene and the 3′ LTR.

Virus assay. Virus was harvested from virus-producing cells by incubating dishes of confluent cells with fresh medium for 16 h, removing the medium, and subjecting it to centrifugation at 3,000 × *g* for 5 min to remove cells and debris. The virus was either assayed immediately or frozen at −75°C for later use. For assay of virus carrying selectable markers, recipient cells were seeded at 5×10^5 per 60-mm dish on day 1. On day 2 the medium was changed to one containing 4 μg of Polybrene per ml, and aliquots of test virus were added. On day 3 the cells were split 1:10 into a selective medium containing 2 mg of G418 (about 50% active) per ml for *neo* virus or 400 μg of hygromycin B (950 U/mg) per ml for *hph* virus. Colonies were stained and counted on day 9. Helper virus was measured by using the S^+L^- assay as previously described (18).

Construction of mutant viruses. To make mutations in the region containing the *gag* start codon (see Fig. 4), a *Pst*I fragment of MoMuLV containing the *gag* start codon was cloned into an M13 vector (15). The fragment was sequenced by the dideoxy chain termination method (22), and one base-pair difference was noted between the published (27) and observed sequences, a G-to-A change at base 617 just upstream of the *gag* start codon. Site-directed mutagenesis was performed by using either of two oligomers, 5′-TCTGGCCCTAATTTTCAG-3′ (M1) or 5′-AACAGTCTAG CCCATAT-3′ (M2), and mutated clones were screened by hybridization with the oligomer used for mutagenesis (32). Several of the mutant clones were sequenced by the dideoxy chain termination method (22), and the appropriate sequence throughout the insert was confirmed. The altered fragments were then reintroduced into pLNL-XHC, which was used because of convenient restriction sites in this plasmid. Specific hybridization of the oligomers to plasmid DNA confirmed the presence of the appropriate mutations in the final plasmids. In addition, the mutation induced by oligomer M2 destroyed a *Hae*III site originally present in pLNL-XHC (see Fig. 4), and this change was also confirmed.

Preparation of viral RNA. Virus-producing cells were seeded at 5×10^6 per 10-cm dish on day 1. The cells were fed on day 2 with 10 ml of medium per 10-cm dish, and virus-containing medium was harvested 12 h later. The cells were refed, and virus was harvested again 12 h later. The virus-containing medium was centrifuged at 3,000 × *g* for 5 min to remove cells and debris, and about 30 ml was carefully overlaid onto 4 ml of 20% sucrose in phosphate-buffered saline containing (per liter) 0.1 g of $CaCl_2$, 0.2 g of KCl, 0.2 g of KH_2PO_4, 0.1 g of $MgCl_2 \cdot 6H_2O$, 8.0 g of NaCl, and 2.16 g of $Na_2HPO_4 \cdot 7H_2O$ (pH 7.5) in a centrifuge tube. RNase was inactivated in the sucrose and other solutions used for RNA preparation by exposure to 0.1% diethylpyrocarbonate overnight followed by autoclaving (15 min, liquid cycle) to remove the remaining diethylpyrocarbonate. The samples were centrifuged at 26,000 rpm for 2.5 h in an SW28 rotor (Beckman). The culture medium and sucrose were then carefully aspirated to avoid contamination of the viral pellet with culture medium. The virus pellet was suspended in 400 μl of TNE (150 mM NaCl, 1 mM EDTA, 10 mM Tris [pH 7.5]) by a scraping procedure, 20 μl of 10% sodium dodecyl sulfate and 40 μl of 3 M sodium acetate (pH 5.2) were added, and the solution was extracted twice with 200 μl of phenol–200 μl of chloroform and once with 400 μl of chloroform. The RNA was precipitated at −70°C after addition of 1 ml of ethanol, centrifuged at 13,000 × *g* for 15 min at 4°C, and washed with 70% cold ethanol. The purified viral RNA was dried under vacuum and suspended in 100 μl of water.

RESULTS

Retrovirus vectors. The retrovirus vectors used (Fig. 1) contain coding regions of the bacterial neomycin (*neo*) or hygromycin B (*hph*) phosphotransferase gene inserted between retroviral LTRs. These dominant-acting selectable genes are expressed by using transcription control signals present in the LTRs. All vectors also contain a previously characterized region called ψ (Fig. 1) which is involved in packaging of genomic RNA into virions. Deletion of the ψ

site from MoMuLV helper virus (MoMuLV bases 215 to 565 [27]) results in very inefficient packaging of genomic RNA into virions (14). In addition to the ψ site, the gag^+ vectors N2 and LHL contain additional MoMuLV sequences (MoMuLV bases 566 to 1038) between the ψ site and the selectable gene. These additional sequences include the start codon and a portion of the *gag* coding region of MoMuLV. The selectable genes in LNL2 and LHL2 are translated from unspliced mRNAs, whereas the selectable genes in N2 and LHL are translated from spliced mRNAs.

Effect of *gag* region inclusion on virus titer. The retrovirus packaging cell line PA317 (16) allows production of all of the virus vectors described above in the absence of helper virus. PA317 cells producing each of the vectors were made as previously described (19). Briefly, plasmid forms of the vectors were transfected into ψ-2 retrovirus packaging cells (14), and virus was harvested 2 days later and used to infect PA317 cells. The infected PA317 cells were exposed to drug-containing medium to select for the presence of the vector. About eight drug-resistant clones for each vector were then screened for virus production, and the best virus producer was selected. PA317 cells containing gag^+ vectors produced 50- to 200-fold-higher virus titers than did PA317 cells containing equivalent gag^- vectors (Table 1). In addition, most of the PA317 clones containing a given vector produced virus titers that were within 10-fold of the highest titer achieved (data not shown); thus, the titer of virus produced by the highest-titer producers is a meaningful measurement of the ability of a vector to produce virus.

Correlation of virus titer with concentration of viral RNA in virus-containing medium. To determine the mechanism underlying the large difference in virus titers produced by PA317 cells containing gag^+ vectors in comparison with PA317 cells containing gag^- vectors, we first examined viral RNA present in medium exposed to virus-producing cells. Virus-containing medium was harvested from cultures of PA317 cells producing each of the vectors, and the virus was purified by centrifugation through 20% sucrose. Viral RNA was prepared from the virus, subjected to electrophoresis, and analyzed by using radioactive probes which hybridized to the drug resistance genes in the vectors (Fig. 2). RNA of the size expected for genomic RNA was detected for each of the vectors. The RNA species for LHL2 did not appear with the exposure time used but was detected after longer exposures (Fig. 2, arrowhead). A dramatic difference in genomic RNA content was observed; medium exposed to cells secreting the gag^+ vectors LHL and N2 contained much more genomic RNA than did medium exposed to cells secreting the corresponding gag^- vectors LHL2 and LNL2. In addition, when medium containing gag^- and gag^+ vectors was mixed before preparation and analysis of the virion RNA, no evidence was seen for unequal RNA loss during purification (Fig. 2). For example, with a mixture of LHL2 and LHL viruses (Fig. 2, lane 3) there was still a clear difference between the amounts of viral RNA present, i.e., a band with the mobility of LHL2 should have been visible in the mixture at the position indicated by the arrowhead, but this band was seen only with a much longer exposure. To quantitate the difference, the major radioactive bands were cut out of the nitrocellulose filter by using the autoradiograph as a guide, and the amount of radioactivity in each band was measured by scintillation counting. The results of this analysis are shown in Table 1 along with the titer for each of the viruses. The ratio of the titers of gag^+ and gag^- vectors corresponds closely to the ratio of genomic viral RNAs. This suggests that the difference between gag^+ and gag^- vectors is manifested at the level of virus production and not at the level of virus entry into or expression in recipient cells.

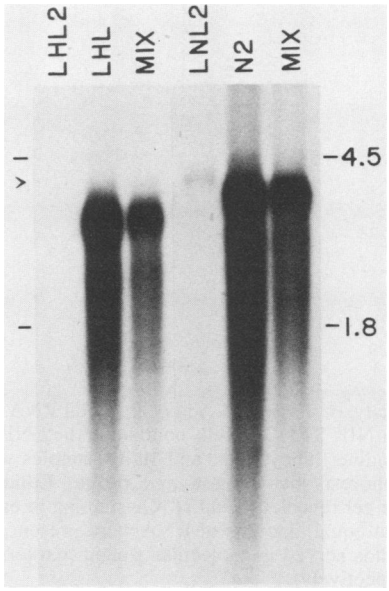

FIG. 2. Analysis of viral RNA in medium exposed to virus-producing cells. Viral RNA in culture medium was purified as described in Materials and Methods, subjected to electrophoresis in agar containing formaldehyde, transferred to nitrocellulose, and hybridized to an *hph* probe (three left lanes) or a *neo* probe (three right lanes), and the bound probe was analyzed by autoradiography. Molecular weight markers were 28S and 18S rRNA (4.5 and 1.8 kilobases, respectively) electrophoresed in adjacent lanes. Lanes labeled MIX represent equal mixtures of virus-containing medium (lane 3, LHL2 plus LHL; lane 6, LNL2 plus N2) which were also subjected to analysis as a control for possible unequal RNA losses during purification. All samples were prepared in parallel, and equal fractions of the purified RNAs, corresponding to about 6 ml of virus-containing medium, were loaded in each lane. The RNA species for LHL2 (arrowhead) did not appear with the exposure time used but was detected after longer exposures.

TABLE 1. Comparison of virus titers with amounts of viral RNA in medium[a]

Virus-producing cells	Virus titer		Amt of viral RNA	
	(CFU/ml)	Ratio	(cpm)	Ratio
PA317/N2 c11	1×10^7	50×	9,600	38×
PA317/LNL2 c2	2×10^5		255	
PA317/LHL c3	2×10^6	200×	7,400	220×
PA317/LHL2 c5	1×10^4		33	

[a] Viral RNA purified from medium exposed to virus-producing cells was subjected to electrophoresis in agarose and transferred to nitrocellulose, and bound RNA was hybridized with radioactive probes by standard methods. Major radioactive bands corresponding to viral genomic RNA were cut out of the filters by using an autoradiograph of the radioactive filter as a guide. The radioactivity in each band was quantitated after addition of 2 ml of scintillation fluid to each filter piece. In addition, a similar size nonradioactive portion of the filter was analyzed by scintillation counting, and reported counts per minute are corrected for this background rate. The bands were cut out of the same filter used for the autoradiograph shown in Fig. 2. The specific activities of the *neo* and *hph* probes were not necessarily the same.

Similarity of vector RNA levels in cells. We examined the levels of viral RNA in the vector-producing PA317 cells to determine whether differences in genomic RNA levels could account for the increased virus production from PA317 cells

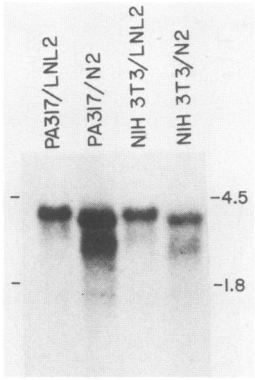

FIG. 3. Analysis of viral RNA in cells. Total RNA was prepared from PA317 or NIH 3T3 TK$^-$ cells containing the LNL2 or N2 virus by using guanidine thiocyanate, and 10-μg samples were analyzed after electrophoresis by using a *neo* probe. Ethidium bromide staining of the gel revealed equal rRNA staining in each lane, thus confirming that equal amounts of RNA were present. The 28S and 18S rRNA bands served as molecular weight markers (4.5 and 1.8 kilobases, respectively).

harboring *gag*$^+$ vectors in comparison with that from cells harboring *gag*$^-$ vectors. PA317 cells containing the *gag*$^-$ LNL2 virus expressed a single message which hybridized to a *neo* probe (Fig. 3, lane 1) and was the same size as the genomic RNA (results not shown). PA317 cells containing the *gag*$^+$ N2 virus expressed two mRNAs which hybridized to a *neo* probe (Fig. 3, lane 2), the larger of which was the same size as the genomic RNA (results not shown); the other represented the spliced message encoding neomycin phosphotransferase. There was only about a twofold-higher level of the genomic message in N2-infected PA317 cells than in LNL2-infected cells. Thus, the small differences in the levels of viral RNA in cells producing these *neo* viruses cannot account for the 50-fold difference in virus production.

We also measured the levels of viral RNA in cells infected with the viral vectors but which did not produce virus particles. Analysis of the RNA species in these cells allowed comparison of mRNA accumulation in the absence of virus production and concomitant genomic RNA export. Virus from PA317 cells producing either vector was used to infect NIH 3T3 TK$^-$ cells at a low multiplicity of infection, and colonies resistant to G418 were selected and pooled. These cell populations were helper virus free, as expected, and contained one copy of either *neo* virus per cell (data not shown). The RNA species in the nonproducer cells which hybridized to a *neo* probe were the same as those found in the PA317 cells (Fig. 3). There was about twofold less genomic RNA in the *gag*$^+$ N2 virus-infected cells compared with that in *gag*$^-$ LNL2-infected cells (Fig. 3, lanes 3 and 4). These results show that RNA levels in cells containing the *gag*$^+$ or *gag*$^-$ vector were similar in cells either secreting or not secreting virus, further supporting the conclusion that differences in viral RNA production in the vector-producing cells are not responsible for the large difference in virus production.

High-titer virus was not due to *trans*-acting proteins. We hypothesized that some protein synthesized from the extra sequences in the *gag*$^+$ vectors was responsible for the increased virus production from packaging cells containing these vectors. This protein could act to more efficiently direct viral genomic RNA into virions. Although the retrovirus packaging cells make all of the viral proteins required to produce transmissible virus from introduced viral vectors, it could be argued that deletion of the ψ site from the helper virus used to make the cells resulted in an inability to make a protein that increases the efficiency of virus production. Introduction of a vector with an intact ψ site and downstream *gag* sequences could allow production of this putative protein.

We tested this hypothesis using a complementation assay in which we introduced a *gag*$^-$ *neo* virus into PA317 cells already containing and producing a *gag*$^+$ *hph* virus. The use of different drug resistance genes in the two viruses allowed independent measurement of virus titers. If a *trans*-acting protein that improved virus RNA packaging was produced by the *gag*$^+$ *hph* virus, the titer of the *gag*$^-$ *neo* virus should have increased. The titer of *hph* virus produced by PA317 cells infected with *gag*$^+$ LHL virus was 3×10^6 CFU/ml and remained essentially unaltered after introduction of *gag*$^-$ LNL2 virus (Table 2). The average titer of LNL2 virus produced by PA317 cells infected with LNL2 and LHL was 8×10^4, essentially the same as the titer produced by PA317 cells infected with the LNL2 virus alone (Table 2). These results exclude the involvement of a *trans*-acting protein in high-titer-virus production.

High-titer virus was not due to *gag*-related proteins. We next considered the possibility that synthesis of some *gag*-related protein acted in *cis* to promote efficient virus production. Such a protein might bind viral RNA shortly after translation and as a result inhibit further translation and provide a signal for packaging of the viral RNA. To test this hypothesis, mutations were made in the *gag* coding region (Fig. 4). Mutation M1 converted the *gag* start codon into a stop codon. A stop codon was used to prevent translation from further upstream, since there is a large open reading frame upstream and in frame with the *gag* reading frame (27). The second mutation, M2, resulted in the insertion of a stop codon two amino acids downstream of the *gag* start codon. Two mutations were made and tested in the hope that at least one of the mutations would not disturb possible viral RNA secondary structure in the region and complicate the

TABLE 2. Lack of complementation between *gag*$^+$ and *gag*$^-$ viruses[a]

Virus-producing cells	Clone	Titer (CFU/ml) of:	
		neo virus	*hph* virus
PA317/LNL2 c2		1×10^5	$<10^3$
PA317/LHL c3		$<10^3$	3×10^6
PA317/LHL c3 infected with LNL2	1	1×10^5	3×10^6
	2	1×10^5	3×10^6
	3	2×10^5	3×10^6
	4	1×10^4	3×10^6
	5	4×10^4	8×10^6
	6	4×10^4	9×10^6
	7	1×10^5	5×10^6
	8	6×10^4	5×10^6
	9	6×10^4	4×10^6
Avg for clones		8×10^4	5×10^6

[a] ψ-2 cells were transfected with the pLNL2 *neo*-virus plasmid, and virus was harvested 2 days later and used to infect PA317/LHL c3 cells as previously described (19). The infected cells were exposed to selective medium containing G418, and drug-resistant clones were isolated by using cloning rings. The clones were analyzed for virus production by using NIH 3T3 TK$^-$ cells as recipients for virus infection and by dividing the infected cells into medium containing G418 and medium containing hygromycin B to quantitate *neo* virus and *hph* virus, respectively. Helper virus production from all cells was also monitored, and all were helper virus free (less than one focus-forming unit per milliliter).

results. Subtle mutations (especially M2) were used to avoid interruption of possible secondary structure.

We tested the effects of these mutations on virus production by introducing viruses containing the mutations into PA317 cells and measuring the production of virus from clones and pools of infected cells. There was no difference in virus production from cells containing either mutation compared with that from cells containing the parental virus (Table 3). Clones producing virus titers of at least 10^7 were obtained by using either mutant or the parental virus. No helper virus was produced (less than one per milliliter) by any of the virus-producing cells. Thus, inhibition of protein synthesis from or through the normal MoMuLV *gag* initiator codon did not affect the production of high-titer virus.

In addition to production of the normal $Pr65^{gag}$ polyprotein, a glycosylated *gag* polyprotein ($gPr80^{gag}$) is made by MoMuLV and other murine leukemia viruses. Translation of the glycosylated *gag* protein begins in a large open reading frame upstream of the normal *gag* start codon and continues downstream and in frame with the normal *gag* protein (5, 6, 23). Translation of the $gPr80^{gag}$ protein is terminated in LNL-M1 and LNL-M2 near the $Pr65^{gag}$ start codon, but this does not preclude production of the amino terminus of $gPr80^{gag}$. Although MoMuLV deletion mutants which do not make $gPr80^{gag}$ are replication competent and are apparently similar to MoMuLV in in vitro assays (24), we wanted to rule out involvement of this region in production of high-titer virus. The 5′ portion of MoMSV is similar to that of MoMuLV, but MoMSV does not make a glycosylated *gag* protein because of small alterations in the region upstream of *gag*, including a frameshift mutation (27). To test the involvement of a glycosylated *gag* protein in high-titer virus production, we inserted this region of MoMSV into pLNL-M1 in place of the corresponding MoMuLV sequences (MoMuLV bases 1 to 568) to make pLNL6 (Fig. 1). LNL6 was introduced into PA317 cells, and G418-resistant cells were analyzed for virus production (Table 3). Populations of PA317/LNL-M1 or PA317/LNL6 cells produced virus with similar titers and individual clones producing at least 10^7 G418-resistant CFU/ml were obtained from PA317 cells containing either vector. Thus, alteration of a gag^+ virus so that it could not make a glycosylated *gag* protein did not affect the titer of virus produced. The results also show that the MoMSV ψ site is functionally identical to that of MoMuLV when introduced into a gag^+ vector. In conclusion, these results rule out the involvement of a *gag*-related protein in high-titer-virus production from gag^+ vectors.

High-titer virus was not related to the presence of splice signals. The gag^+ vectors N2 and LHL (Fig. 1) rely on splicing for the production of mRNAs that can be translated to produce neomycin phosphotransferase. Presumably, the full-length viral message cannot be translated to produce neomycin phosphotransferase because translation would begin at the intervening *gag* start codon. In contrast, the normal retrovirus splice donor was removed from the gag^- vectors LNL2 and LHL2 (Fig. 1) because in these vectors the full-length viral mRNA is translated to yield neomycin phosphotransferase, and use of the splice donor could only result in a reduction of this message and a possible reduction in the amount of neomycin phosphotransferase produced. These design considerations also lead to the possibility that the presence of the splice donor was in part responsible for high-titer-virus production from the gag^+ viruses. To test this possibility, the splice donor in LNL6 was removed by replacing it with the corresponding region from LNL2 to make LNL6(SD^-). We used LNL6 for this test because it does not contain the start codon for *gag*; therefore, translation of neomycin phosphotransferase from the full-length viral message would be more likely than in a vector contain-

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                620  HaeIII
                 •        •
MoMLV     CTGAAAATATGGGCCAGACTGTT
                 **
LNL-M1    CTGAAAATTAGGGCCAGACTGTT
                   *
LNL-M2    CTGAAAATATGGGCTAGACTGTT
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FIG. 4. Mutant viruses. The MoMuLV wild-type sequence around the *gag* start codon (underlined sequence) present in the LNL-XHC virus was altered as shown to generate the mutant viruses LNL-M1 and LNL-M2. The sequence in this region for this isolate of MoMuLV differs from the published sequence (27) at base 617. Alterations are indicated by asterisks, and the MoMuLV base number is indicated.

TABLE 3. Effect of alterations in the *gag* reading frame on virus production[a]

Virus-producing cells and clone	Titer (CFU/ml)
PA317/LNL-M1	
1	3×10^6
2	4×10^6
3	6×10^5
4	3×10^6
5	4×10^6
6	5×10^6
7	3×10^6
8	1×10^7
Mixture	6×10^6
PA317/LNL-M2	
1	4×10^6
2	8×10^6
3	4×10^5
4	6×10^6
5	1×10^7
6	2×10^4
Mixture	8×10^6
PA317/LNL-XHC	
1	1×10^6
2	3×10^6
3	8×10^6
4	1×10^7
5	1×10^6
Mixture	6×10^6
PA317/LNL6	
4	1×10^6
5	1×10^6
6	4×10^5
7	$<10^4$
8	2×10^7
9	2×10^6
10	7×10^6
Mixture	8×10^6

[a] The *neo* virus in pLNL-XHC is identical to N2 except for a different linker between the *neo* gene and the 3′ LTR. The *gag* mutations M1 and M2 were introduced into pLNL-XHC. The plasmid pLNL6 was made by replacing the MoMuLV ψ site in pLNL-M1 with that of MoMSV. Virus was made from each of the virus-containing plasmids by transfection of ψ-2 cells and was used to infect PA317 cells as previously described (19). Infected PA317 cells were exposed to G418, and drug-resistant clones containing each *neo* virus were isolated. A mixture of >100 clones was also analyzed. Clones and mixtures were assayed for production of *neo* virus and helper virus. No helper virus was detected (less than one per milliliter).

ing the codon, such as N2. PA317 cells producing LNL6(SD$^-$) were generated, and a mixture of G418-resistant clones was screened for virus production. The virus titer was 5×10^6 Neo^r CFU/ml, which is similar to the titer obtained for a mixture of PA317 clones containing LNL6 (8×10^6; Table 3). This shows that the presence of the splice donor in the *gag*$^+$ vectors was not responsible for high-titer-virus production.

DISCUSSION

We have observed that certain retroviral vectors yield higher-titer virus and promote more efficient infection of several important cell types than do earlier vector designs. The major structural difference between the two types of vectors is the presence of *gag* sequences in the high-titer vectors. In this study we used representatives from both the *gag*$^-$ and *gag*$^+$ vector classes to examine the basis for the difference in phenotype. As expected, retrovirus packaging cells containing the *gag*$^+$ vectors produced virus at a titer at least 1 order of magnitude higher than that produced by packaging cells containing the *gag*$^-$ viruses. This increase was entirely accounted for by a proportionally larger amount of viral genomic RNA in *gag*$^+$ virus stocks. The amounts of viral RNA present in cells producing either type of vector were similar, eliminating a role for elements such as transcriptional enhancers that could increase the amount of viral RNA available for export from cells producing the vectors. It was concluded that vector-derived proteins, especially *gag*-related proteins, were not involved in this effect, based on several criteria: (i) *gag*$^+$ vectors did not complement *gag*$^-$ vectors when introduced into the same packaging cell line, excluding a role for *trans*-acting proteins; (ii) stop codons inserted into the *gag* coding region did not affect the production of high-titer virus; and (iii) further disruption of the large open reading frame upstream of the *gag* start codon by replacement of the MoMuLV ψ site with that of MoMSV had no effect on virus titer. These results, especially the lack of complementation of *gag*$^-$ vectors by *gag*$^+$ vectors, make it unlikely that a protein encoded in the short open reading frames left in the altered vectors had any role in high-titer-virus production. Therefore, the higher titer of the *gag*$^+$ vectors was due to *cis*-acting element(s) in the viral genome which promoted more efficient packaging of this RNA into virions than did those of *gag*$^-$ vectors.

Previous studies have identified a sequence (ψ) between MoMuLV bases 215 and 565 that is required for packaging of MoMuLV genomic RNA. The important element of this signal has been further localized to the 5′ end of ψ between MoMuLV bases 215 and 355 (13, 24). However, data presented here show that the ψ signal is not sufficient for efficient packaging of viral RNA. Since the presence of the *gag* region was the major difference between the high- and low-titer viruses examined and since the difference in titers correlated with the efficiency of packaging of viral RNA, we conclude that the signal for efficient packaging of viral RNA extends into the *gag* region of MoMuLV.

The *gag*$^+$ vector N2 was part of a series of vectors containing various amounts of *gag* sequences which were tested for efficiency of gene transfer (D. Armentano and E. Gilboa, unpublished results). Inclusion of additional *gag* sequences not present in N2 did not lead to a significant further increase in vector titer. Elimination of the *gag* sequences led to a reduction in titer similar to that observed here. Thus, the signal required for efficient viral RNA packaging, which we will call ψ^+, is contained within MoMuLV sequences from bases 215 to 1039. The finding that sequences within ψ^+ can be deleted from MoMuLV helper virus (MoMuLV bases 400 to 454, 402 to 484, or 537 to 602) while preserving the replicative ability of the virus (24) allows division of ψ^+ into two domains. The 5′ domain (ψ) is required for packaging of viral RNA, whereas the 3′ domain is not required but promotes more efficient packaging.

In addition to the N2 vector, the *gag*$^+$ vector SDHT (19) yields high-titer virus which is similar to N2 in its ability to efficiently infect cells (9, 12). The viral sequences in SDHT were derived from spleen focus-forming virus, which is closely related to its associated helper virus, Friend murine leukemia virus. We have found that small deletions and other minor alterations in the portion of *gag* within the ψ^+ site of SDHT result in less efficient virus production from the vector (unpublished results). Thus, the conclusions that we reached concerning the extent of the MoMuLV packaging signal are likely to be more generally applicable.

Analogous to the ψ signal of MoMuLV, packaging signals located between the 5′ LTR and the start of the *gag* gene have been found in RSV (11, 25) and in spleen necrosis virus (29), both of which are avian retroviruses, and in the amphotropic murine retrovirus 4070 (26). In addition, a packaging signal has been identified in the amino-terminal sequence of the *gag* gene of RSV (21), and a similar signal appears to overlap the amino-terminal sequence of *gag* in spleen necrosis virus (29). The splice donor for the *env* mRNA in RSV lies in the 5′ end of *gag* upstream of the packaging signal in *gag*; thus, the biological significance of this packaging signal probably involves the exclusion of the *env* mRNA from virions. Since the *env* splice donor in spleen necrosis virus (30) and MoMuLV (27) lies upstream of the most 5′ packaging signal, no simple explanation for the presence of an additional packaging signal in the *gag* region is apparent.

It is formally possible that the function of the extra *gag* sequences is purely to insulate the real packaging signal from the effects of surrounding RNA and that they simply act as a spacer that has no direct involvement in packaging of viral RNA. Although we cannot exclude this possibility, it seems unlikely considering the number of vectors that have been constructed with various genes juxtaposed 3′ of the ψ site, including the LNL2 and LHL2 vectors examined here, all of which apparently yield virus at titers lower than do vectors containing the ψ^+ site. It would be expected that not all sequences would inhibit the function of the ψ site in viral RNA packaging.

Introduction of all retroviral constructions described here into PA317 cells resulted in production of helper-virus-free vectors. In every assay for production of a viral vector, we screened 1 ml of the same virus-containing medium and never detected helper virus. In contrast, we have shown that introduction of the *gag*$^+$ vector N2 into ψ-2 or PA12 cells results in helper virus production which is apparently due to a recombination event between sequences upstream of and including *gag* sequences which are common to both the vector and the mutant helper virus used to make the packaging cells (19). Although the mutant helper virus used to make the PA317 cells contains a similar overlapping region, it has additional alterations which prevent helper virus production (16). We have tested for helper virus production from PA12 cells infected with the mutant vectors LNL-M1 and LNL-M2 to determine whether the stop codons inserted into the *gag* region would prevent helper virus production. Although helper virus production is reduced by using LNL-

M2 and further reduced by using LNL-M1 (in comparison with LNL-XHC or N2), it is not eliminated (unpublished results). The region of overlap between LNL-M1 and the packaging DNA in which a recombination would lead to production of replication-competent virus is reduced to only 56 base pairs; thus, it is clear that overlap between vector and packaging DNA should be avoided as much as possible to prevent helper virus production.

Acutely transforming retroviruses can arise from recombination of a replication-competent retrovirus with host cell sequences. Such recombinants are usually replication defective. It is interesting that many of the acutely transforming avian and mammalian viruses retain *gag* sequences and often express a transforming gene as a *gag-onc* fusion protein (28). Harvey murine sarcoma virus and Kirsten murine sarcoma virus are exceptions, but these viruses have apparently acquired functionally similar sequences from endogenous mouse VL30 RNAs. In contrast, there are many examples of transforming viruses lacking *pol* or *env* sequences. These findings may be partially explained by the presence of a packaging function in the *gag* sequences which results in selection for viruses containing these sequences.

The vectors described here that contain mutations to prevent *gag*-related protein synthesis should be useful for gene transfer into animals and potentially in gene therapy. Glycosylated *gag* proteins made by MoMuLV helper virus appear on the surface of and are secreted by MoMuLV-infected cells. These proteins are highly antigenic in animals and lead to an immune response against infected cells. Vectors such as N2 may also express portions of glycosylated *gag* proteins; thus, cells infected with this vector may be antigenic after introduction into animals. LNL6 has deletions and mutations which should prevent *gag*-related protein synthesis and the associated antigenicity of infected cells. Reduced antigenicity and the efficient gene transfer achieved with the LNL6 vector will allow reevaluation of the cause of low-level gene expression in animals after gene transfer using retroviruses.

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